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The Evaluation of Placental Inflammation via the Genomic Inflammatory Index (GII) in Relation to Key Perinatal Factors

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ABSTRACT:

Inflammation *in utero* is associated with a variety of adverse health outcomes experienced by the offspring, such as forms of cerebral palsy and autism. Identifying and understanding what may be causing the *in utero* inflammation is vital for attempting to mitigate exposure to inflammation by the fetus. The objective of this study was to create a genomic inflammatory index (GII) that can be utilized to effectively evaluate and quantify inflammation levels in the placenta. For this study, 386 placentas from the Extremely Low Gestational Age Newborn (ELGAN) cohort, with sufficient data on mRNA levels, were assessed. The GII consists of a score derived from 14 inflammation-related genes selected for their established role in pro-inflammatory signaling pathways. The GII showed variance in placental inflammatory levels across subjects. Crude and adjusted models were used to examine the relationship between 47 perinatal factors and the GII. Significant differences in inflammatory levels indicated by the GII were observed between Black mothers and White mothers, and between those who took steroids and those who did not take steroids while in labor at the hospital. It is anticipated that the GII will provide a novel genomics tool for quantifying placental inflammation, allowing for further research on causes of inflammation in the placenta.

ACKNOWLEDGEMENTS

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INTRODUCTION:

The placenta is a vital organ for fetal development that undergoes changes throughout the entire span of pregnancy. The placenta begins to develop after fertilization, emerging from the outer wall of the blastocyst. The placenta serves a variety of roles, including hormone production, oxygen and nutrient transfer, and waste elimination, while also acting as a barrier against the mother's immune system, chemical toxicants, and xenobiotics (1). Overall, the health of the fetus is largely dependent upon the proper functioning of the placenta (1, 2). Due to the placenta's integral role in development, placental physiology has been tied to lifelong effects on the offspring (1, 2). In fact, phenotypes of the placenta are predictive of chronic conditions experienced later-in-life by the fetus, such as coronary heart disease and Hodgkin's lymphoma (3-5).

The placenta is of particular interest to the growing field of the developmental origins of health and disease hypothesis (DOHaD). DOHaD explores the later-in-life health effects associated with exposures by the fetus and neonate (6, 7). When the placenta is analyzed, it can be excised from the maternal or fetal side with different biological interpretations. Since the fetal side of the placenta is derived from the zygote, the fetus and the villous chorion share the same genetic composition (8). The placenta can serve as an unique indicator of what the fetus was exposed to *in utero* as well as the resulting gene expression and epigenetic alterations (9-16).

In animal models, intrauterine inflammation has been tied to modified neurobehavioral functioning of the offspring, altered white matter development in the brain, acute brain injury, disruption of serotonin regulation, and altered gene expression (17-20). Adverse impacts of *in utero* inflammation have also been found in human cohorts, with data from the Extremely Low Gestational Age Newborn (ELGAN) cohort reporting that inflamed placentas were associated

with an increased risk of developing diparetic cerebral palsy later in life (at ~24 months post-term equivalent) (21). Additional studies also found associations between prenatal inflammatory exposure and cerebral palsy in both preterm and term cohorts (22, 23). A cohort of children born at the New York Methodist Hospital demonstrated an association between acute placental inflammation and an increased risk for autism spectrum disorder (24). Combined, preterm birth and chorioamnionitis were found to increase the odds ratio for attention deficit hyperactivity disorder (ADHD) and neurodevelopmental disabilities in children (25). Overall, exposure to inflammatory stimuli is likely accountable for, or greatly contributes to, the development of a vast number of adverse health outcomes in childhood and later life.

In utero inflammation and its precursors may be evaluated through the placenta. The presence of acute chorioamnionitis, and other histological markers of inflammation, is used as an indicator of intra-amniotic inflammation (26). Identifying acute chorioamnionitis involves histological evaluation of the amnion, chorionic connective tissue, or chorionic plate for abundantly distributed infiltration of both fetal and maternal neutrophils (27, 28). Histological evaluations of inflammation are quite useful in terms of determining whether or not there is inflammation in the placenta, however, the effectiveness of using histological evaluations for determining varying grades of inflammation has been questioned (26, 27). Alternatively, exposure to inflammatory stimuli can be identified by assessing the upregulation of proinflammatory genes, through an analysis of mRNA levels, such as the tumor necrosis factor-α (*TNF-α*) (29-31). Potential causes of *in utero* inflammation have been identified, with maternal demographics, such as obesity and infections, being the major perpetrators (26, 32-35). Of concern is the association between *in utero* inflammatory exposure and a variety of adverse health outcomes experienced by the offspring as summarized above.

The first aim of this study was to develop a tool for quantifying exposure to inflammation in the placenta in order to address shortcomings with popular histological evaluations of inflammation. Using the ELGAN study cohort, a Genomic Inflammatory Index (GII) was developed by assessing the mRNA expression within the placenta. The GII represents a composite measure of pro-inflammatory gene expression across fourteen genes. The second aim of this study was to use the GII to identify modifiable and non-modifiable maternal antecedents associated with increased pro-inflammatory gene expression. Understanding which maternal antecedents significantly impact inflammatory gene expression can inform intervention efforts to both prevent and decrease inflammation in the placenta, with potential positive impacts on later child health.

METHODS

The ELGAN Cohort

Cohort characteristics and study design have been detailed extensively elsewhere (36). Enrollment into the original ELGAN cohort began in 2002, and continued until 2004. Participants consist of infants born prior to 28 weeks' gestation (n = 1506) and their mothers (n = 1249). Pregnant women at 14 medical centers in the United States were recruited, with the Institutional Review Board at each medical center approving of the study. Maternal consent for participation was granted either upon admission to the hospital, or shortly after giving birth. Demographic and pre-pregnancy variables were collected after birth by a trained research nurse using a structured questionnaire. Additional information was also collected using medical records.

The perinatal factors identified in this study were gathered from the maternal medical records and the maternal interview. Antecedents of interest consisted of non-modifiable and modifiable perinatal factors previously analyzed for their association with positive child health, with the addition of more maternal medications and diseases screened for in the maternal interview (37, 38). In total, 47 antecedents were identified, with 10 maternal demographic antecedents, 19 maternal medication antecedents, 14 maternal diseases, and 4 maternal medical conditions (**Supplement 1**).

Placenta Tissue Collection

Of the 1249 ELGAN cohort mothers, 450 donated placentas. From these, 386 placentas had sufficient demographic data, passed quality assessment/quality control (QA/QC) steps described below, and were analyzed for mRNA expression.

As detailed previously, placentas were biopsied within hours of delivery (39, 40). In brief, the appropriate sampling area was identified and the amnion was removed to reveal the chorion. Less than one gram of the base of the chorion, consisting of fetally derived tissue, was collected into a 2 mL cryogenic vial. The vials were placed in liquid nitrogen and stored at -80 °C before and after being shipped to the University of North Carolina at Chapel Hill (UNC). For processing, 0.2 g was sliced from the sample and washed with 1x phosphate-buffered saline. Lysed tissue for nucleic acid extraction was formed by homogenizing the slices with lysis buffer and Buffer RLT (Qiagen, Germantown, MD).

mRNA Extraction and Sequencing

The AllPrep DNA/RNA/miRNA Universal kit (Qiagen) was used to isolate RNA molecules greater than 18 nucleotides in length. RNA quality and quantity were determined using LabChip (Perkin Elmer) RNA integrity scores and DV200 values. A QuantSeq 3′ mRNA-Seq Library Prep Kit (Lexogen) was used to conduct a genome-wide mRNA expression analysis. One lane of the Illumina Hiseq 2500 was used to sequence and pool (single-end 50 bp) RNA-sequencing libraries, which were prepared using Sciclone G3 (Perkin Elmer). The sequencing reads produced by the Illumina Hiseq 2500 were aligned to the GENCODE database v30, and arranged with Salmon (version 0.11.3) (41, 42). In total there were 37,268 unique human RNA transcripts used for further analysis.

Normalization of Count Data and Generation of GII

Aligned count data were used for data processing and statistical analyses. Count data were first filtered to exclude universally lowly expressed transcripts, requiring that > 25% of the

samples be expressed at signals above the overall median signal intensity, as done in previous genome-wide mRNA analyses (43-47). This resulted in a total of 10,408 mRNA transcripts. Of the 390 placentas evaluated, two samples were removed due to having all zero counts. Quality assessment and quality control (QA/QC) was conducted on the count data using both 1) calculation and visualization of principal components via the prcomp function, and 2) hierarchical clustering, including calculation of distance metrics and visualization, using the hclust function. Following removal of two outliers, the final analysis sample of the same n=386 subjects was constructed. The DESeq2 package (v1.24.0) was used to normalize the count data using median signal intensity across all 10,408 genes (48).

Identifying Candidate Genes to Comprise the GII

The "HALLMARK_INFLAMMATORY_RESPONSE" gene set (Hallmark Gene Set, n = 200), version 5.0, provided by the GSEA and MSigDB Team was utilized as a baseline list of inflammation related genes (49-51). The entire list of detectable genes above the low expression cut-off (n = 10,408) in the ELGAN placentas was compared to the Hallmark Gene Set to determine direct overlap between the two sets. A subsequent list of 102 genes was formed (**Supplement 2).** Using the searchable human gene database, GeneCards, each of the 102 genes were evaluated for being pro-inflammatory or anti-inflammatory (52). Specifically, only the "Summaries" and "Function" sections of GeneCards were used to evaluate inflammatory functions related to each gene. If an inflammatory relationship was mentioned, but the relationship was not specified as pro- or anti- inflammatory, the gene was further analyzed using PubMed. A final list of fourteen pro-inflammatory related genes met our criteria. These fourteen genes are: C-C motif chemokine ligand 2 (*CCL2*); CD14 molecule (*CD14*); chemerin

chemokine-like receptor 1 (*CMKLR1*); colony stimulating factor 1 (*CSF1*); eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2); coagulation factor III, tissue factor (*F3*); interleukin 18 receptor 1 (*IL18R1*); interleukin 1 receptor type 1 (*IL1R1*); interleukin 4 receptor (*IL4R*); interferon regulatory factor 1 (*IRF1*); lysophosphatidic acid receptor 1 (*LPAR1*); oxidized low density lipoprotein receptor 1 (*OLR1*); toll like receptor 2 (*TLR2*); toll like receptor 3 (*TLR3*).

The normalized mRNA counts of the n=14 proinflammatory genes identified above for n=386 subjects were extracted. A genomic inflammatory index (GII) was calculated as follows: GII= log[sum(count of all 14 normalized genes)+1]. One unit was added to each subjects' mRNA normalized counts due to ten subjects having a sum of zero, since log of zero is undefined. All of the above statistical analyses were conducted in R (v3.6.1).

Evaluation of the GII in Relation to Perinatal Factors

Demographic antecedents were coded as follows: non-ordinal categorical variables included race (White, Black, Asian, Native American, Mixed race, Other), maternal age (<21, 21-35, 35+ at the time of birth), insurance status (HMO/private insurance, self-pay, public insurance, no insurance), and pre-pregnancy BMI (Underweight, normal, overweight, obese). Ordinal categorical variables included maternal education (1: ≤ 12, 2: 13-15, 3: ≥16) and plurality (1,2,3+). Binary variables included Hispanic ethnicity (yes/no), marital status (married/ not married), smoked during pregnancy (yes/no), use of IVF for the index pregnancy (yes/no), and fetal sex (male/female). Maternal diseases/conditions and medications were coded as follows: binary variables included use of NSAID during pregnancy (yes/no); use of antibiotic during pregnancy (yes/no); use of aspirin during pregnancy (yes/no); cerclage (yes/no;, any

cerebral spinal fluid infection during pregnancy (yes/no); use of antibiotic during current hospital admission (yes/no); use of antenatal corticosteroid (ACS) during current hospital admission (yes/no); use of magnesium sulfate (for preeclampsia/ pregnancy-induced hypertension) during current hospital admission (yes/no); use of magnesium sulfate (for tocolysis) during current hospital admission (yes/no); use of insulin during current hospital admission (yes/no); use of steroids (not for promoting lung maturity) during current hospital admission (yes/no); presence of sepsis during current hospital admission (yes/no); maternal discharge for pregnancy-induced hypertension/preeclampsia/toxemia (yes/no); maternal discharge for hemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome (yes/no), discharge diagnosis of chorionitis/chorioamnionitis (yes/no). Continuous variables included highest temperature during the hospital admission for giving birth and highest white blood cell count during the hospital admission for giving birth. Where there was data for the presence of a condition, and condition-specific medication use, a three-level non-ordinal variable was created: pre-pregnancy asthma (no, yes and took medication, yes and took no medication), during pregnancy UTI (no, yes and took medication, yes and took no medication), during pregnancy proteinuria (no, yes and took medication, yes and took no medication), during pregnancy STD (no, yes and took medication, yes and took no medication), during pregnancy vaginal infection (no, yes and took medication, yes and took no medication), during pregnancy fever (no, yes and took medication, yes and took no medication), during pregnancy flu (no, yes and took medication, yes and took no medication), during pregnancy pneumonia (no, yes and took medication, yes and took no medication), during pregnancy toxemia (no, yes and took medication, yes and took no medication), and pre-pregnancy diabetes (no, yes and took medication, yes and took no medication). Clinical and histological evaluations of inflammation were coded as: grade of chorion/decidua inflammation

(none-minimal/mild-severe), any grade of inflammation in the chorion or amnion (yes/no), and moderate to severe indicators of inflammation in the chorionic plate or chorion or umbilical cord or fetal stem vessels [CCUFV] (yes/no).

Linear regression models were fit with the GII as the dependent variable and various antecedents as the independent variable, thus resulting in a beta estimate representing the mean difference in GII comparing index to reference group of the antecedent. First, unadjusted models were run to examine crude relationships between GII and maternal antecedents. Significant relationships were defined a $p<0.05$ for the mean difference in GII estimate. If the crude estimate for an antecedent was significant adjusted models were run controlling for maternal BMI, maternal insurance, fetal sex, smoking during pregnancy, maternal marital status and maternal race. Covariates were selected for their known relationship to placental inflammation or significance in crude models (53-55). In adjusted models, significance was also defined as $p<0.05$ for the mean difference in GII estimate. Regression modelling was conducted in SAS (SAS software v.9.4) utilizing the PROC GENMOD procedure.

RESULTS

Study Population Characteristics

This cohort of 386 women included 235 (61.5%) White women, 112 (29.3%) Black women, 5 (1.3%) Asian women, 4 (1.1%) Native American women, 12 (3.1%) mixed race women, and 14 (3.7%) women who identified their race as "Other" on the maternal questionnaire. The majority (91.7%) of mother's identified as non-Hispanic. Just over two-thirds (67.4%) of the cohort were between 21 and 35 years old, with an almost equal number of women having either completed their education up to and including 12th grade (39.2%) or completed at least a college degree (38.4%). The first and second most common insurance types were HMO or Private (65.3%) and Public Insurance (31.8%) respectively. A majority of the women (57%) were not married at the time of birth, and the most common BMI was in the Normal category (53.4%). About ten percent (10.8%) of the women had smoked during pregnancy. A total of 226 of the women had a singleton gestation in their participatory pregnancy, with 55 women utilizing IVF or ICSI for conception of their participatory pregnancy (**Table 1**).

Table 1. Study Population Characteristics (n=386) of ELGAN Cohort Mother's with Placental Samples Analyzed for mRNA Expression

	N (%)* (Total n=386)	GII			
		Median	Min	Max	IQR
Race					
White	235 (61.5%)	5.05	0	8.98	2.25
Black	112 (29.3%)	5.92	0	8.61	2.35
Asian	5 (1.3%)	5.57	3.03	5.99	1.69
Native American	4 (1.1%)	5.04	3.90	6.91	0.776
Mixed	12 (3.1%)	5.79	3.13	7.32	2.40
Other	14 (3.7%)	4.97	2.00	6.88	1.20
Missing	4				
Hispanic					
No	354 (91.7%)	5.26	0	8.98	2.43
Yes	32 (8.3%)	5.33	2.00	7.94	2.49
Maternal Age (Years)					
<21	44 (11.4%)	5.64	0	8.47	2.53

21-35	260 (67.4%)	5.20	0	8.98	2.49
>35	82 (21.2%)	5.25	0	7.86	2.12
Maternal Education (Years of Education)					
≤ 12	147 (39.2%)	5.43	0	8.30	2.72
13-15	84 (22.4%)	5.19	0	8.47	2.34
≥ 16	144 (38.4%)	5.14	0	8.98	1.14
Missing	11				
Insurance					
HMO or Private	247 (65.3%)	5.17	0	8.98	2.32
Self-Pay	5 (1.3%)	5.35	2.74	5.63	0.716
Public Insurance	120 (31.8%)	5.67	0	8.47	2.62
No Insurance	6 (1.6%)	5.22	3.19	6.57	1.86
Missing	8				
Marital Status					
Not Married	166 (43.0%)	5.58	0	8.61	2.69
Married	220 (57.0%)	5.15	0	8.98	2.13
Pre-Pregnancy BMI					
Underweight	26 (7.0%)	4.42	1.19	7.15	3.16
Normal	199 (53.4%)	5.38	0	8.98	2.55
Overweight	67 (17.9%)	5.40	0	7.34	1.90
Obese	81 (21.7%)	5.19	0	8.47	2.20
Missing	13				
Smoking During Pregnancy					
No	338 (89.2%)	5.20	0	8.98	2.32
Yes	41 (10.8%)	5.56	0	8.30	2.18
Missing	7				
Plurality					
1	226 (61.1%)	5.42	0	8.61	2.59
2	118 (31.9%)	5.10	0	8.98	2.09
3+	26 (7.0%)	5.23	0	7.85	2.13
Missing	16				
IVF or ICSI					
No	41 (42.7%)	4.95	0	7.12	1.57
Yes	55 (57.3%)	4.81	0	8.98	1.95
Missing	290				

*Percentage of non-missing values

GII and the Study Population

The placentas in this study displayed a range of mRNA expression across the 14 genes used to calculate the GII (**Figure 1**). In addition, hierarchical clustering showed that the genes *IL4R* and

EIF2AK2 had the most similar z-score standardized mRNA counts across subjects (**Figure 1**). The GII values ranged from 0 to 8.98 with the data from all 386 women being slightly negatively skewed. The median GII was 5.29 (**Figure 2**).

An examination of the 14 genes and their expression for the ELGAN subject with the maximum GII (8.98) showed the highest expression for *F3* (**Figure 3A)**. *F3* is involved with blood coagulation cascades and its expression can be induced by mediators of inflammation such as cytokines and endotoxins (52). Additionally, the subject with the maximum GII had a greater z-score standardized mRNA count than at least 50% of the other subjects for all but one of the genes, *IRF1*, with *F3* and *LPAR1* being over eight standard deviations greater than the mean GII (**Figure 3B**). Meanwhile, the subject with the minimum GII had all zero counts for each of the fourteen GII genes (**Supplement 3**).

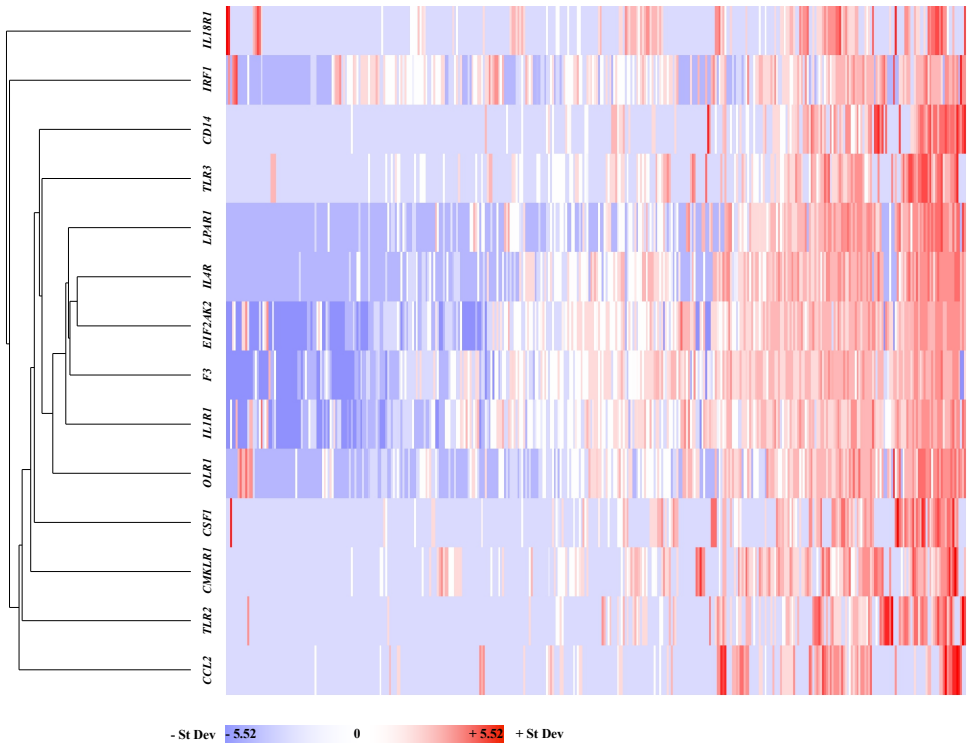

Figure 1. Heat map of z-score standardized mRNA counts. This heat map illustrates the z-score standardized mRNA counts for each of the genes used to calculate the GII across all subjects (n = 386). Data are z-score standardized for each gene, and hierarchical clustering of

genes and subjects was performed. Blue represents a lower relative expression level, while red represents a higher relative expression level. Genes are represented on the y axis and subjects on the x axis.

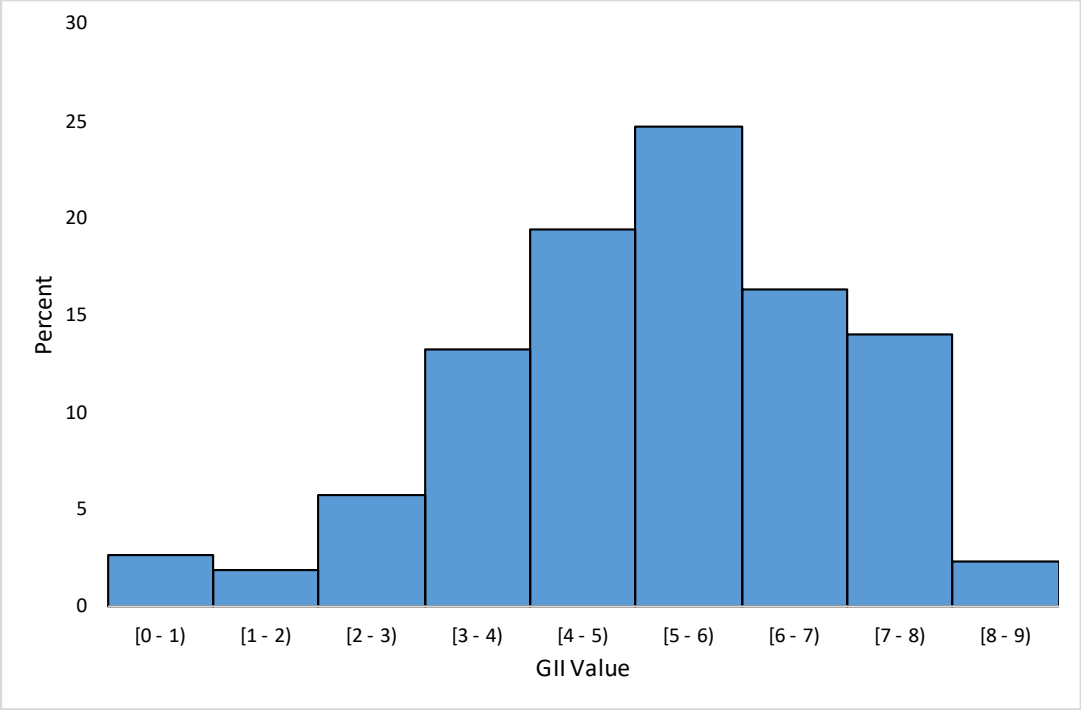

Figure 2. Histogram showing the distribution of the GII for all 386 subjects. GII bins are marketed by "[" showing an included value, and ")" showing the non-included upper value of the bin (ie. with [0-1) the bin is comprised of any value including zero, up to, and not including, one).

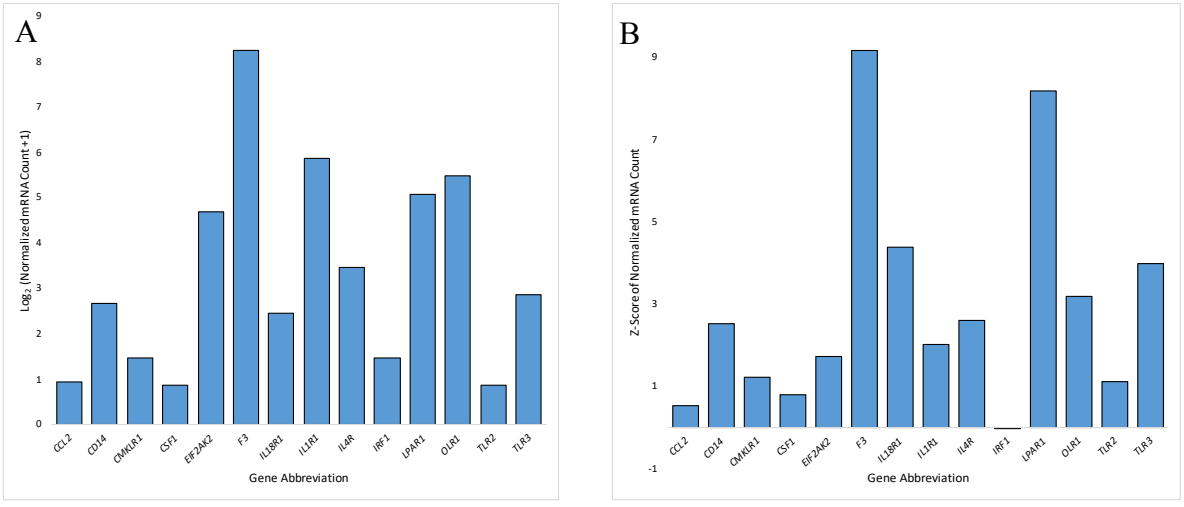

Figure 3.
A: Bar chart of the $\log_2$ (Normalized mRNA Count + 1) for each of the 14 genes from the subject with the largest GII.
B: Bar chart of the z-score standardized mRNA counts for the subject with the largest GII.

Maternal Antecedents / Perinatal Factors:

A total of 47 different perinatal factors were examined in relation to the GII. Those that displayed any significant association (either in the crude or adjusted models) are detailed below. The results of all comparisons can be found in the Supplemental Materials (**Supplement 4**).

GII and Marriage

In the unadjusted models the placental GII for mothers who were married was on average 0.4102 counts (on a log scale; beta value = 0.4102) lower than the GII of mothers who were not married during pregnancy (p=0.0486; **Figure 4A; Table 2**). In the models adjusting for maternal BMI, maternal insurance, fetal sex, smoking during pregnancy, and maternal race, the mean difference in GII for marital status was no longer significant, with a beta value of -0.2981 and p-value of 0.2431 (**Table 2**).

GII and Fever

The placental GII for mothers who had a fever during pregnancy and took fever medications was on average 0.8528 counts (on a log scale) lower than the GII of mothers who did not have a fever during pregnancy, and this mean difference was statistically significant (p=0.0486) in the unadjusted models (**Figure 4B; Table 2**). In the adjusted models there was no longer a significant difference for any of the fever classifications (**Table 2**). The comparison of those who had a fever and did not take medications versus those who did not have a fever was insignificant in both the unadjusted and adjusted models (p= 0.3952 and p=0.3905, respectively; **Table 2**).

GII and Steroids

A significant mean difference in GII was found between women who were given steroid(s) (not for promoting lung maturity) when they were in the hospital giving birth versus women who were not given such steroids in both the adjusted and unadjusted models (p=0.0423 and p=0.023, respectively; **Figure 4C; Table 2**). The beta values in the unadjusted and adjusted models were 0.6753 and 0.7501, respectively, signifying that those who were given a steroid had a higher GII on average (**Table 2**).

GII and Race

In both the unadjusted and adjusted models the placental GII for mothers who identified as Black was on average 0.8701 and 0.9707 counts (on a log scale) higher, respectively, than the GII of mothers who identified as White (p<0.001; **Figure 4D; Table 2**).

Table 2. Beta values, standard error and associated p-values of crude linear regressions and adjusted multivariate linear regressions of GII onto maternal antecedents. Only maternal antecedents that were significant in the unadjusted modeling are shown. Beta values represent the predicted change of GII between the chosen reference subgroup and the remaining subgroups of a maternal antecedent category. The reference subgroup for race was those who identified as white, the reference subgroup for steroid was those who did not receive a steroid (not for promoting lung maturity) during the hospital admission for giving birth, the reference subgroup for marriage was those who were married, and the reference subgroup for fever was those who did not report having a fever during pregnancy. Adjusted for maternal BMI, maternal insurance, fetal sex, smoking during pregnancy, maternal marital status, and maternal race where appropriate.

	Mean GII	Unadjusted			Adjusted		
		Beta Value	Standard Error	p-Value	Beta Value	Standard Error	p-Value
Race							
White	4.90	-	-	-	-	-	-
Black	5.77	0.8701	0.196	**<0.001**	0.9707	0.2367	**<0.0001**
Steroid*							
No	5.08	-	-	-	-	-	-
Yes	5.76	0.6753	0.3325	**0.0423**	0.7501	0.33	**0.023**
Marital Status							
Not Married	5.39	-	-	-	-	-	-

Married	4.98	-0.4102	0.1787	**0.0217**	-0.2981	0.2554	0.2431
Fever During Pregnancy							
No	5.18	-	-	-	-	-	-
Yes (No Meds)	4.12	-1.0515	1.2366	0.3952	-1.0417	1.2132	0.3905
Yes (Meds)	4.33	-0.8538	0.4329	**0.0486**	-0.5897	0.4416	0.1817
*Not for Promoting Lung Maturity							

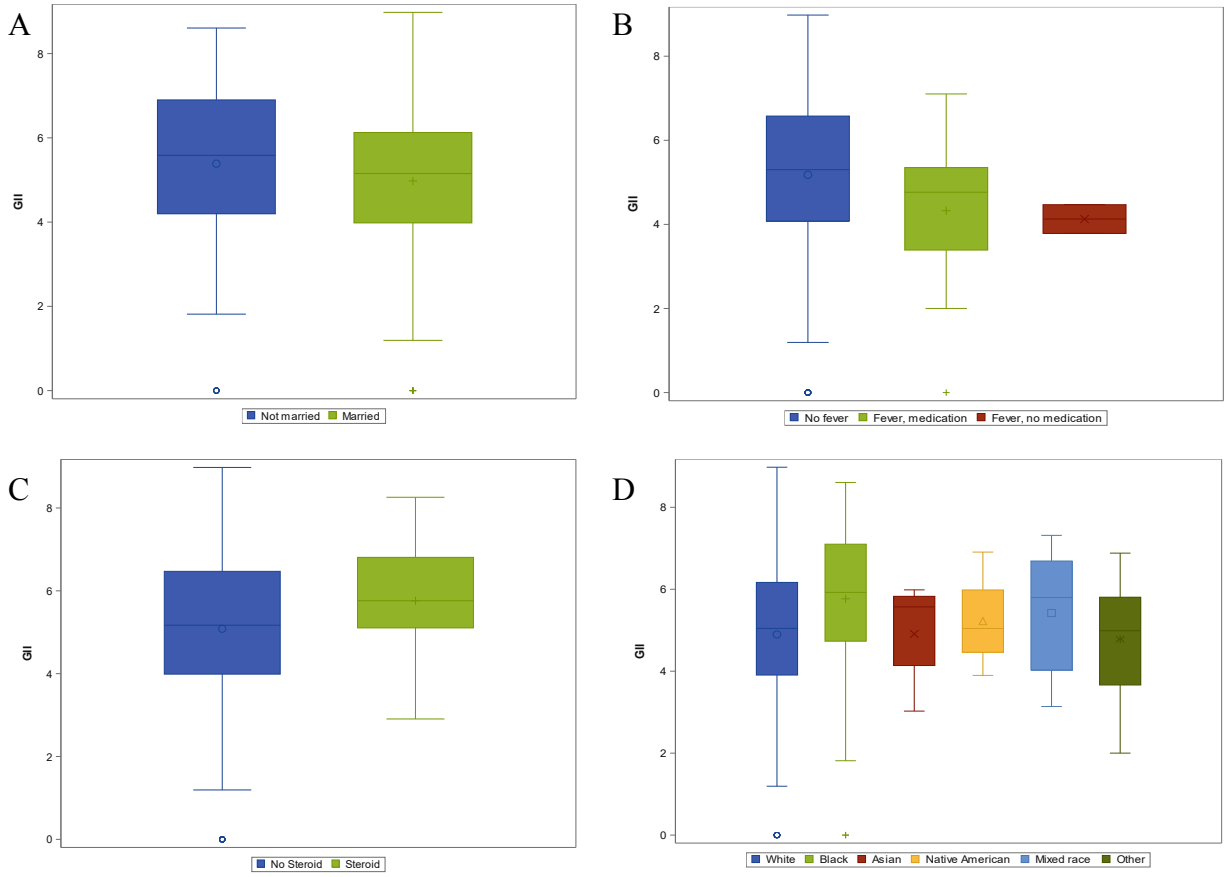

Figure 4 A – D. Boxplots of unadjusted results that were statistically significant. 4A) Boxplot of the GII for those who were married and not married. 4B) Boxplot of the GII for different fever classifications during pregnancy. 4C) Boxplot of the GII for those who were administered a steroid (not for lung maturity) while giving birth versus those were not. 4D) Boxplot of the GII for each race recorded.

GII and Clinical/Histological Indicators of Inflammation

We next set out to compare the GII values to other measures of inflammation in placentas. There was not a significant mean difference in GII between cases of any inflammation or high grades of inflammation versus cases of low grade inflammation or no inflammation in the following clinical and histological indicators of inflammation: Chorion/Decidua Grade of Inflammation

(beta value= 0.096, p=0.6066), Any Inflammation in the Chorion or Amnion (beta value= 0.0114, p=0.9512), Moderate to Severe Indicators of Inflammation in Any of the CCUFV (beta value= 0.0644, p=0.7346; **Table 3**). While not statistically significant, each of the aforementioned clinical indicators of inflammation did have positive beta values in relation to GII, indicating a positive trend in GII scores for cases with any inflammation or high grades of inflammation. In addition, all but one of the markers of inflammation, and their subcategories, did not have direct overlap, showing variability in their measures (**Table 4**). Only the moderate to severe classification of inflammation in the chorion/decidua had a precise overlap with there being any marker of inflammation in the chorion or amnion. This is shown by 0% of the subjects who were marked as having moderate to severe chorion/decidua inflammation being recorded as no inflammation in the chorion or amnion for the respective variables in a cross tabulation (**Table 4**).

Table 3. Beta values, standard error and associated p-values of crude linear regression models for histological and clinical markers of inflammation in the placenta. These markers included the grade of chorion/decidua inflammation, any grade of inflammation in the chorion or amnion, and moderate to severe indicators of inflammation in the chorionic plate or chorion or umbilical cord or fetal stem vessels [CCUFV].

	Mean GII	Unadjusted		
		Beta Value	Standard Error	p-Value
Chorion/Decidua Grade of Inflammation				
None to Minimal	5.12	-	-	-
Mild to Severe	5.21	0.096	0.1864	0.6066
Any Inflammation in the Chorion or Amnion				
No	5.15	-	-	-
Yes	5.16	0.0114	0.1857	0.9512
Moderate to Severe Indicators of Inflammation in Any of the CCUFV				
No	5.12	-	-	-
Yes	5.19	0.0644	0.1901	0.7346

Table 4. Cross tabulation of the histological and clinical markers of inflammation in the placenta.

Key: Frequency Percent		Chorion/Decidua Grade of Inflammation			Any Inflammation in the Chorion or Amnion		
		None-Minimal	Moderate-Severe	Total	No	Yes	Total
Any Inflammation in the Chorion or Amnion	No	189 52.21%	**0** **0%**	189 52.21%			
	Yes	15 4.14%	158 43.65%	173 47.79%			
	Total	204 56.35%	158 43.65%	362 100%			
Moderate to Severe Indicators of Inflammation in Any of the CCUFV	No	200 54.95%	27 7.42%	227 62.36%	185 51.1%	40 11.05%	225 62.15%
	Yes	6 1.65%	131 35.99%	137 37.64%	4 1.1%	133 36.74%	137 37.85%
	Total	206 56.59%	158 43.41%	364 100%	189 52.21%	173 47.79%	362 100%

DISCUSSION

Exposure to inflammatory stimuli *in utero* is associated with a variety of adverse later in life health outcomes (21-25, 56). It is a critical undertaking to understand what causes inflammation *in utero,* especially when considering maternal factors that are potentially modifiable. Currently, *in utero* inflammation is mainly evaluated through histological identification of inflammation, with quantification of the mRNA levels of inflammation related genes being an underused resource (26-31). In the present study we set out to develop an index that could quantify the presence of inflammation in the placenta at the transcriptome level and be used to evaluate antecedents of placental inflammation. There were three main findings: 1) the GII demonstrated a strong ability to capture variance in pro-inflammatory gene expression, 2) significant differences in inflammation detected via the GII were found for potential maternal antecedents of placental inflammation, and 3) there was a non-significant positive trend between the GII and histological markers of inflammation, with the histological markers of inflammation demonstrating inconsistencies in their determination.

In evaluating the GII, it was important to examine the extent to which the GII varied between subjects. A heat map of the mRNA count per gene by subject, and a model of the distribution of GIIs, were used to analyze the variance of GII within the cohort. In summary, both the heat map and the distribution of GIIs showed a range of GIIs, with a minimum of 0 and a maximum of 8.98, indicating differences in pro-inflammatory gene expression across the placentas analyzed. These results are consistent with current evaluations of placental inflammation used in the literature in that a) placental inflammation can be detected and analyzed and b) there are alternate levels, or amounts, of *in utero* inflammation (26-35). With this variance in GIIs comes the question as to what were the driving forces behind these

differences in the transcriptome level measure of inflammation. In the literature, maternal demographic factors, infections, and diseases are associated with placental inflammation, leading to an analysis of what maternal demographic factors and diseases experienced during pregnancy were associated with an increased GII in the present cohort (26, 32-35).

Two maternal antecedents were found to have a statistically significant mean difference in GII between subjects in only the unadjusted models. Being married significantly lowered the subjects' GIIs in the unadjusted models, shown by a negative beta value, potentially signifying a protective effect of being married. Previous studies have shown a protective effect of being married on overall health and inflammation levels, albeit, other studies have warned of the importance of considering the quality of the marriage on overall health as well (57-60). In addition, a negative beta value was produced when comparing the GIIs of those with no fevers during pregnancy versus those who took fever medication for fevers that occurred during pregnancy, possibly showing an association between taking fever medications and a reduction in GII. Such a reduction in GII after taking fever medications for a fever could result from fever medications such as NSAIDs having anti-inflammatory affects. However, Tylenol is also a popular fever medication that does not have anti-inflammatory affects, highlighting the importance of knowing which specific type of fever medication these mothers took. Since in the adjusted models the associations between the GII, marital status, and fever classification were no longer significant, the relationships seen were likely driven by the confounders controlled for in the adjusted models, including insurance status and race.

Two perinatal factors remained significant in relation to the GII in both the unadjusted and adjusted models: steroid use and race. Those who received a steroid (not for promoting lung maturity) while admitted for delivery had greater GIIs on average compared to mothers who did

not receive such steroids, signifying an increase in inflammation amongst those who received the steroids. As specified, these steroids were not for promoting lung maturity and instead were possibly given for flare-ups in asthma or autoimmune conditions such as lupus or rheumatoid arthritis. In which case the mothers may have been given prednisone or prednisolone: anti-inflammatory corticoid steroids typically used for treating maternal inflammatory diseases (61, 62). Even though the exact condition as for why the steroids were administered was not specified, the steroids were most likely supposed to have an anti-inflammatory effect which was not seen in the present study. What may have driven the increase in GII in those given steroids is the timing and reactionary use of the steroid, where the steroid was likely administered in response to an inflammatory event. At that point, inflammation related mRNA levels may have already been high and remained high as the placenta was removed, preventing the steroids from normalizing the inflammation levels.

Another factor possibly accounting for the increased GII in those administered steroids during birth is where the steroids were metabolized and what their target was. Through pharmacokinetics, drugs are administered based on their metabolization and movement throughout the body. Drugs such as dexamethasone, which is given to promote fetal lung maturity, are known to not be metabolized by the placenta, allowing them to reach the fetus at high concentrations (61-63). Meanwhile, drugs such as prednisone and prednisolone do not reach the fetus at as high of concentrations, and are thus used to treat maternal diseases instead (61, 62, 64). Since the steroids administered to the mothers were not for promoting lung maturity, their anti-inflammatory effects may not have reached the fetal side of the placenta where the samples used in this study were collected from. Thus, allowing for the fetal side of the placenta to remain inflamed despite the mothers being given steroids.

The maternal demographic, race, also remained statistically significant in the adjusted models. Specifically, Black mothers on average had a higher GII than White mothers. Racial disparities in placental pathology have been reported in previous studies (53, 54). For example, one study found that Black mothers of preterm infants had a higher adjusted odds of having chronic inflammation in the placenta at birth than White mothers (54). Proposed driving forces behind the differences in placental inflammation between White and Black mothers includes a mix of socioeconomic status, racial discrimination, stress levels, and the weathering hypothesis, which states that cumulative socioeconomic disadvantage has a deteriorating effect on the physical health of African American women (54, 65-67). Further research into the association between race and placental inflammation is warranted.

Three clinical and histological evaluations of inflammation at birth in the ELGAN cohort were assessed using the GII: grade of chorion/decidua inflammation, any grade of inflammation in the chorion or amnion, and moderate to severe indicators of inflammation in the chorionic plate or chorion or umbilical cord or fetal stem vessels [CCUFV]. While there was no statistically significant increase in GII's between mothers with no or low grades of inflammation versus mothers with inflammation or high grades of inflammation, there was a slightly positive trend. Interestingly, when comparing the clinical and histological evaluations of inflammation against one another there was not a direct overlap between a majority of like categories. This shows variability amongst the clinical/histological variables and possible inconsistencies in measurement, which have previously been reported in the literature (26, 27). A study by Redline et al. evaluated how well pathologists' identifications of placental inflammatory lesions agreed. Overall, there was high agreement when simply determining if there was inflammation or not, however, when quantifying the grade or stage of inflammation the agreement became weak (27).

These results demonstrate the limitations of histological measurements and a need for alternative methods for quantifying inflammation. The lack of association between the clinical and histological evaluations of inflammation and the GII, and slight discrepancies in the clinical/histological evaluations themselves, calls for further investigation into these clinical/histological variables.

The GII has strengths as a novel tool to evaluate inflammation in the placenta and has various ways to be improved. One strength of the GII is how the GII is a continuous and quantitative variable, allowing for a gradient in inflammation to be formed that could be used to analyze how varying levels of inflammation are related to antecedents of inflammation. Meanwhile, histological and clinical assessments of inflammation are ordinal and qualitative variables, causing less flexibility in analysis. A second major strength of the GII includes how the GII is comprised of mRNA transcript levels, versus histological evaluations, and is thus not subject to as grave of human error and opinion as chorioamnionitis may be (26, 27). Still, the results from this study and the GII in its current form are not without limitations. The ELGAN cohort consists of only extremely preterm newborns, which may limit generalizability of the GII. In addition, a few of the antecedents gathered from the maternal interview may be subject to social desirability bias, such as smoking during pregnancy. Another form of inconsistency may result from mothers not accurately recalling what diseases they had or what medications they took during pregnancy. Certain results may have been affected by low sample sizes, such as many of the diseases and medications reported, due to mothers not answering that portion of the survey or not having contracted such diseases during pregnancy. One limitation of the GII is how the mRNA levels may be representative of one point in time, in this case at birth, since gene expression and transcription can change throughout life. Histological determination of inflammation is also

subject to such a limit due to the change in protein levels through protein synthesis and degradation. Further work on the GII is warranted as certain antecedents associated with placental inflammation in the literature, such as maternal BMI, were not observed as causing a significant difference in GII in the present study (26, 32-35, 68).

In summary, the GII represents a composite measure of pro-inflammatory gene expression across fourteen genes that was used to assess the influence of maternal antecedents on placental pro-inflammatory gene expression. Future directions and improvement plans for the GII include expanding the GII gene list to include additional genes used to evaluate inflammation, such as: caspase-1 (*CASP1*) and toll-like receptor 4 (*TLR4*) (35, 69, 70). These genes were originally not included in the GII because they were not found to be direct matches between the Hallmark Gene Set and the detectable mRNAs expressed in the ELGAN cohort placentas. A second improvement will involve adding additional socioeconomic variables as confounding variables to better understand what may be driving results to be significant or not in adjusted and unadjusted models. A final future direction will involve an in-depth comparison of the GII to chorioamnionitis and other inflammation variables collected form the ELGAN cohort, in addition to understanding and resolving any disconnect between the GII and the inflammatory variables. Overall, the long term goal is for the GII to serve as a biomarker of *in utero* inflammatory exposure with hopes of mitigating exposure to inflammatory stimuli and better understanding which infants are at the highest risk for developing diseases later in life.

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SUPPLEMENTAL MATERIALS

Supplement 1. Table of Antecedents

Maternal Demographics	**Maternal Medications (DP = During Pregnancy; HAB = Hospital Admission for Birth)**	**Maternal Diseases (HAB = Hospital Admission for Birth)**	**Other Medical Conditions (HAB = Hospital Admission for Birth)**
1. Racial Identity 2. Hispanic 3. Maternal Age 4. Maternal Education 5. Single Martial Status 6. Insurance Status 7. Pre-pregnancy BMI 8. Smoking While Pregnant 9. Plurality 10. IVF or ICSI	1. DP antibiotics 2. DP NSAID 3. DP aspirin 4. Pre-pregnancy asthma medication 5. DP UTI medication 6. DP proteinuria medication 7. DP STD medication 8. DP vaginal infection medication 9. DP fever medication 10. DP flu medication 11. DP pneumonia medication 12. Pre-pregnancy diabetes medication 13. DP toxemia medication 14. HAB Antibiotic 15. HAB Antenatal Corticosteroid (ACS) 16. HAB Magnesium Sulfate (for Preeclampsia / Pregnancy-induced Hypertension) 17. HAB Magnesium Sulfate (for Tocolysis) 18. HAB Insulin 19. HAB Steroid (Not for Promoting Lung Maturity)	1. CSF infection 2. Pre-pregnancy Asthma 3. UTI 4. STD 5. Vaginal infection 6. Fever 7. Flu 8. Pneumonia 9. Pre-pregnancy Diabetes 10. Toxemia 11. HAB Highest Temperature 12. HAB Sepsis 13. HAB Maternal Discharge for Pregnancy-induced Hypertension / Preeclampsia / Toxemia 14. HAB Maternal Discharge for HELLP Syndrome	1. Cerclage 2. Proteinuria 3. HAB Highest White Blood Cell Count 4. HAB Chorionitis/ Chorioamnionitis

Supplement 2. List of genes that directly overlapped between the Hallmark Gene Set and the ELGAN detectable genes in the placenta (n = 102)

Genes in both Hallmark Gene Set and ELGAN Gene Set	
A	*'ABCA1' 'ABI1' 'ACVR1B' 'ACVR2A' 'ADM' 'AHR' 'APLNR' 'ATP2A2' 'ATP2B1' 'ATP2C1' 'AXL'*
B	*'BEST1' 'BTG2'*
C	*'CALCRL' 'CCL2' 'CD14' 'CD55' 'CD82' 'CDKN1A' 'CMKLR1' 'CSF1' 'CSF3R' 'CXCR6' 'CYBB'*
D	*'DCBLD2'*
E	*'EDN1' 'EIF2AK2'*
F	*'F3'*
G	*'GABBR1' 'GNAI3' 'GPC3' 'GPR183'*
H	*'HBEGF' 'HIF1A' 'HRH1'*
I	*'ICOSLG' 'IFNAR1' 'IFNGR2' 'IL10RA' 'IL15RA' 'IL18R1' 'IL1R1' 'IL4R' 'INHBA' 'IRF1' 'ITGA5' 'ITGB8'*
K	*'KCNJ2' 'KIF1B' 'KLF6'*
L	*'LCP2' 'LDLR' 'LPAR1' 'LYN'*
M	*'MET' 'MMP14' 'MSR1' 'MXD1' 'MYC'*
N	*'NAMPT' 'NFKB1' 'NFKBIA' 'NMI' 'NPFFR2'*
O	*'OLR1' 'OSMR'*
P	*'P2RX4' 'P2RX7' 'PDE4B' 'PDPN' 'PLAUR' 'PSEN1' 'PTPRE' 'PVR'*
R	*'RAF1' 'RASGRP1' 'RELA' 'RIPK2' 'RNF144B'*
S	*'SCARF1' 'SELENOS' 'SEMA4D' 'SERPINE1' 'SGMS2' 'SLC11A2' 'SLC1A2' 'SLC28A2' 'SLC31A1' 'SLC31A2' 'SLC4A4' 'SLC7A1' 'SLC7A2' 'SRI' 'STAB1'*
T	*'TAPBP' 'TIMP1' 'TLR2' 'TLR3' 'TNFRSF1B' 'TNFSF10' 'TNFSF15' 'TPBG'*

Supplement 3. Normalized mRNA counts per gene and GII for the subject with the minimum GII and for the subject with the maximum GII

Subj.	**Gene**														
	CCL2	***CD14***	***CMKLR1***	***CSF1***	***EIF2AK2***	***F3***	***IL18R1***	***IL1R1***	***IL4R***	***IRF1***	***LPAR1***	***OLR1***	***TLR2***	***TLR3***	***GII***
1	0.910	6.37	2.73	1.82	25.5	301	5.46	58.3	10.9	2.73	33.7	44.6	1.82	7.28	8.98
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Supplement 4. Beta values, standard error and associated p-values of crude linear regressions and adjusted multivariate linear regressions of the GII for all maternal antecedents

	Mean GII	Unadjusted			Adjusted		
		Beta Value	Standard Error	p-Value	Beta Value	Standard Error	p-Value
Maternal Demographics							
Race							
White	4.90	-	-	-	-	-	-
Black	5.77	0.8701	0.196	**<0.001**	0.9707	0.2367	**<0.0001**
Asian	4.91	0.0126	0.7713	0.987	0.2583	0.7691	0.737
Native American	5.22	0.326	0.8606	0.7048	0.5311	0.8971	0.5538
Mixed	5.42	0.5228	0.5051	0.3006	0.567	0.5191	0.2747
Other	4.78	-0.117	0.4695	0.8032	-0.1434	0.5002	0.7743
Hispanic							
No	5.15	-	-	-	-	-	-
Yes	5.24	0.0922	0.323	0.7752	-	-	-
Maternal Age (Years)							
<21	5.32	0.2225	0.285	0.4349	-	-	-
21-35	5.10	-	-	-	-	-	-
>35	5.23	0.1297	0.2214	0.5579	-	-	-
Maternal Education (Years of Education)							
≤ 12	5.17	-	-	-	-	-	-
13-15	5.29	-0.0735	0.1029	0.4754	-	-	-
≥ 16	5.02	-0.0735	0.1029	0.4754	-	-	-
Insurance							
HMO or Private	5.12	-	-	-	-	-	-
Self-Pay	4.77	-0.347	0.7907	0.6608	-	-	-
Public Insurance	5.25	0.1281	0.1948	0.5109	-	-	-
No Insurance	4.83	-0.2929	0.7233	0.6855	-	-	-
Marital Status							
Not Married	5.39	-	-	-	-	-	-
Married	4.98	-0.4102	0.1787	**0.0217**	-0.2981	0.2554	0.2431
Pre-Pregnancy BMI							
Underweight	4.71	-0.4389	0.364	0.2279	-	-	-
Normal	5.15	-	-	-	-	-	-
Overweight	5.06	-0.0866	0.2465	0.7255	-	-	-
Obese	5.33	0.1865	0.23	0.4177	-	-	-
Smoking During Pregnancy							
No	5.11	-	-	-	-	-	-
Yes	5.31	0.1998	0.289	0.4893	-	-	-

Plurality							
1	5.18	0.0941	0.2005	0.6387	-	-	-
2	5.09	-	-	-	-	-	-
3+	5.16	0.0775	0.3824	0.8393	-	-	-
IVF or ICSI							
No	4.63	-	-	-	-	-	-
Yes	5.08	0.451	0.3316	0.1738	-	-	-
Maternal Diseases and Subsequent Medications							
Asthma (Pre-Pregnancy)							
No	5.16	-	-	-	-	-	-
Yes (No Meds)	4.70	-0.462	0.495	0.3506	-	-	-
Yes (Meds)	5.05	-0.1124	0.2966	0.7047	-	-	-
UTI							
No	5.14	-	-	-	-	-	-
Yes (No Meds)	6.37	1.4338	1.7554	0.4141	-	-	-
Yes (Meds)	5.06	-0.085	0.2619	0.7456	-	-	-
STD							
No	5.12	-	-	-	-	-	-
Yes (No Meds)	7.03	1.9102	1.24	0.1234	-	-	-
Yes (Meds)	5.29	0.1698	0.4937	0.7308	-	-	-
Vaginal Infection							
No	5.13	-	-	-	-	-	-
Yes (No Meds)	5.45	0.3183	0.7229	0.6597	-	-	-
Yes (Meds)	5.13	-0.0012	0.2526	0.9961	-	-	-
Fever							
No	5.18	-	-	-	-	-	-
Yes (No Meds)	4.13	-1.0515	1.2366	0.3952	-1.0417	1.2132	0.3905
Yes (Meds)	4.33	-0.8538	0.4329	**0.0486**	-0.5897	0.4416	0.1817
Flu							
No	5.18	-	-	-	-	-	-
Yes (No Meds)	4.35	-0.8333	0.4924	0.0906	-	-	-
Yes (Meds)	4.22	-0.9642	0.7179	0.1793	-	-	-
Pneumonia							
No	5.14	-	-	-	-	-	-
Yes (Meds)	4.88	-0.2587	1.017	0.7992	-	-	-
Diabetes (Pre-Pregnancy)							
No	5.13	-	-	-	-	-	-
Yes (No Meds)	7.32	2.1868	1.751	0.2117	-	-	-
Yes (Meds)	5.17	0.0425	0.6249	0.9457	-	-	-
Toxemia							
No	5.15	-	-	-	-	-	-
Yes (No Meds)	4.97	-0.1848	0.4243	0.6632	-	-	-
Yes (Meds)	4.98	-0.1716	0.3859	0.6566	-	-	-
CSF Infection							

No	5.15	-	-	-	-	-	-
Yes	5.22	0.0741	0.3384	0.8267	-	-	-
Highest Temp*							
Change in GII per One Degree Temperature Increase	-	0.0501	0.0953	0.5987	-	-	-
Sepsis*							
No	5.15	-	-	-	-	-	-
Yes	5.84	0.6935	0.8789	0.4301	-	-	-
Maternal Discharge for Pregnancy-induced Hypertension / Preeclampsia / Toxemia*							
No	5.10	-	-	-	-	-	-
Yes	5.43	0.3258	0.2453	0.1842	-	-	-
Maternal Discharge for HELLP Syndrome*							
No	5.17	-	-	-	-	-	-
Yes	4.84	-0.3246	0.4338	0.4544	-	-	-
Other Maternal Medications							
Antibiotics							
No	5.17	-	-	-	-	-	-
Yes	5.05	-0.362	0.2911	0.2137	-	-	-
NSAIDs							
No	5.13	-	-	-	-	-	-
Yes	5.16	-0.0688	0.4406	0.876	-	-	-
Aspirin							
No	5.13	-	-	-	-	-	-
Yes	5.22	-0.2076	0.4818	0.6666	-	-	-
Antibiotics*							
No	5.16	-	-	-	-	-	-
Yes	5.12	0.1206	0.4072	0.7671	-	-	-
Antenatal Corticosteroid (ACS)*							
No	5.35	-	-	-	-	-	-
Yes	5.13	-0.2234	0.2988	0.4547	-	-	-
Magnesium Sulfate (for Preeclampsia /							

Pregnancy-induced Hypertension)*							
No	5.14	-	-	-	-	-	-
Yes	5.13	-0.015	0.2547	0.9532	-	-	-
Magnesium Sulfate (for Tocolysis)*							
No	5.22	-	-	-	-	-	-
Yes	5.09	-0.1349	0.1826	0.4601	-	-	-
Insulin*							
No	5.10	-	-	-	-	-	-
Yes	5.78	0.6862	0.4235	0.1052	-	-	-
Steroid (Not for Promoting Lung Maturity)*							
No	5.08	-	-	-	-	-	-
Yes	5.76	0.6753	0.3325	**0.0423**	0.7501	0.33	**0.023**
Other Maternal Conditions							
Cerclage							
No	5.18	-	-	-	-	-	-
Yes	4.81	-0.3673	0.2801	0.1898	-	-	-
Proteinuria							
No	5.14	-	-	-	-	-	-
Yes (No Meds)	5.17	0.0277	0.2971	0.9257	-	-	-
Yes (Meds)	4.80	-0.341	0.563	0.5447	-	-	-
Highest White Blood Cell Count*							
Change in GII per White Blood Cell Count Increase	-	-0.0112	0.0177	0.5282	-	-	-
Chorionitis/ Chorioamnionitis*							
No	5.11	-	-	-	-	-	-
Yes	5.34	0.227	0.2309	0.3257	-	-	-
**During hospital admission for giving birth							